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(71) Applicant *(for all designated States except US)*: MARS UK LIMITED [GB/GB]; 3D Dundee Road, Slough, Berkshire SL1 4JS (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : SPENCER, Margaret, Elizabeth [GB/GB]; 24 Totley Brook Road, Sheffield S17 3QS (GB). HODGE, Rachel [GB/GB]; 16 Mount Avenue, Leicester LE5 3RN (GB).

(74) Agents: SHEARD, Andrew, Gregory et al.; Kilburn & Strode, 30 John Street, London WC1N 2DD (GB).

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(57) Abstract

A 21 kD protein, and its 23 kD expression precursor, believed to be the source of peptide flavour precursors in cocoa (*Theobroma cacao*) have been identified. Genes coding for them have been probed, identified and sequenced, and recombinant proteins have been synthesised.

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RECOMBINANT 21 kD COCOA PROTEIN AND PRECURSOR

This invention relates to proteins and nucleic acids derived from or otherwise related to cocoa.

The beans of the cocoa plant (*Theobroma cacao*) are the raw material for cocoa, chocolate and natural cocoa and chocolate flavouring. As described by Rohan ("Processing of Raw Cocoa for the Market", FAO/UN (1963)), raw cocoa beans are extracted from the harvested cocoa pod, from which the placenta is normally removed, the beans are then "fermented" for a period of days, during which the beans are killed and a purple pigment is released from the cotyledons. During fermentation "unknown" compounds are formed which on roasting give rise to characteristic cocoa flavour. Rohan suggests that polyphenols and theobromine are implicated in the flavour precursor formation. After fermentation, the beans are dried, during which time the characteristic brown pigment forms, and they are then stored and shipped.

Biehl *et al*, 1982 investigated proteolysis during anaerobic cocoa seed incubation and identified 26kD and 44kD proteins which accumulated during seed ripening and degraded during germination. Biehl asserted that there were storage proteins and suggested that they may give rise to flavour-specific peptides.

Biehl *et al.*, 1985 again asserted that amino acids and peptides were important for flavours.

Fritz *et al*, 1985 identified polypeptides of 20kD and 28kD appearing in the cytoplasmic fraction of cocoa seed extracts at about 100 days after pollination. It appears that the 20kD protein is thought to have glyceryl acyltransferase activity.

Pettipher *et al.*, 1990 suggested that peptides are important for cocoa flavour and refers to 48kD and 28kD storage proteins.

In spite of the uncertainties in the art, as summarised above, proteins apparently responsible for flavour production in cocoa beans have now been identified. Further, it has been discovered that, in spite of Fritz's caution that "cocoa seed mRNA levels are notably low compared to other plants" (*loc. cit.*), it is possible to apply the techniques of recombinant DNA techniques to the production of such proteins.

According to a first aspect of the invention, there is provided a 23kD protein of *Th. cacao* or a fragment thereof.

The 23kD protein may be processed *in vivo* to form a 21kD polypeptide.

According to a second aspect of the invention, there is provided a 21kD protein of *Th. cacao* or a fragment thereof.

The term "fragment" as used herein and as applied to proteins or peptides indicates a sufficient number of amino acid residues are present for the fragment to be useful. Typically, at least four, five, six or even at least 10 or 20 amino acids may be present in a fragment. Useful fragments include those which are the same as or similar or equivalent to those naturally produced during the fermentation phase of cocoa bean processing. It is believed that such fragments take part in Maillard reactions during roasting, to form at least some of the essential flavour components of cocoa.

Proteins in accordance with the invention may be synthetic; they may be chemically synthesised or, preferably, produced by recombinant DNA techniques. Proteins produced by such techniques can therefore be termed "recombinant proteins". Recombinant proteins may be glycosylated or non-glycosylated; non-glycosylated proteins will result from prokaryotic expression systems.

Theobroma cacao has two primary subspecies, *Th. cacao cacao* and *Th. cacao sphaerocarpum*. While proteins in accordance with the invention may be derived from these subspecies, the invention is not limited solely to these subspecies. For example, many cocoa varieties are hybrids between different species; an example of such a hybrid is the trinitario variety.

The invention also relates to nucleic acid, particularly DNA, coding for the proteins referred to above (whether the primary translation products, the processed proteins or fragments). The invention therefore also provides, in further aspects:

- nucleic acid coding for a 23kD protein of *Th. cacao* or for a fragment thereof; and

- nucleic acid coding for a 21kD protein of *Th. cacao* or for a fragment thereof.

Included in the invention is nucleic acid which is degenerate for the wild type protein and which codes for conservative or other non-deleterious mutants. Nucleic acid which hybridises to the wild type material is also included.

Nucleic acid within the scope of the invention will generally be recombinant nucleic acid and may be in isolated form. Frequently, nucleic acid in accordance with the invention will be incorporated into a vector (whether an expression vector or otherwise) such as a plasmid. Suitable expression vectors will contain an appropriate promoter, depending on the intended expression host. For yeast, an appropriate promoter is the yeast pyruvate kinase (PK) promoter; for bacteria an appropriate promoter is a strong lambda promoter.

Expression may be secreted or non-secreted. Secreted expression is preferred, particularly in eukaryotic expression systems; an appropriate signal sequence may be present for this purpose. Signal sequences derived from the expression host (such as that from the yeast alpha-factor in the case of yeast) may be more appropriate than native cocoa signal sequences.

The invention further relates to host cells comprising nucleic acid as described above. Genetic manipulation may for preference take place in prokaryotes. Expression will for preference take place in a food-approved host. The yeast *Saccharomyces cerevisiae* is particularly preferred.

The invention also relates to processes for preparing nucleic acid and protein as described above by nucleic acid replication and expression, respectively.

cDNA in accordance with the invention may be useful not only for obtaining protein expression but also for Restriction Fragment Length Polymorphism (RFLP) studies. In such studies, detectably labelled cDNA (eg radiolabelled) is prepared. DNA of a cultivar under analysis is then prepared and digested with restriction enzymes. Southern blotting with the labelled cDNA may then enable genetic correlations to be made between cultivars. Phenotypic correlations may then be deduced.

The invention will now be illustrated by the following non-limiting examples. The examples refer to the accompanying drawings, in which:

Figure 1 shows a map of a full length cDNA clone hybridising with an oligonucleotide probe for the 21kD protein, together with the regions covered by DNA sequencing;

Figure 2 shows the DNA sequence of cDNA coding for the 21kD protein and the presumed amino-acid sequence of the encoded 23 kD precursor;

Figure 3 shows the relationship between the 21kD protein and trypsin inhibitors from other plants;

Figure 4 shows a map of plasmid pJLA502;

Figure 5 shows two yeast expression vectors useful in the present invention; vector A is designed for internal expression and vector B is designed for secreted expression;

Figure 6a shows, in relation to vector A, part of the yeast pyruvate kinase gene showing the vector A cloning site, and the use of *Hin-Nco* linkers to splice in the 21kD gene;

Figure 6b shows, in relation to vector B, part of the yeast alpha-factor signal sequence showing the vector B cloning site, and the use of *Hin-Nco* linkers to create an in-phase fusion; and

Figure 7 shows a map of plasmids pMY9 and pMY10, referred to in Example 16.

EXAMPLES

Example 1

Identification of the Major Seed Proteins

It is not practicable to extract proteins directly from cocoa beans due to the high fat and polyphenol contents, and proteins were, therefore, extracted from acetone powders made as follows. Mature beans from cocoa of West African origin (*Theobroma cacao amelonada*) were lyophilised and ground roughly in a pestle and mortar. Lipids were extracted by Soxhlet extraction with diethyl ether for two periods of four hours, the beans being dried and further ground

between extractions. Polyphenols and pigments were then removed by several extractions with 80% acetone, 0.1% thioglycollic acid. After extraction the resulting paste was dried under vacuum and ground to a fine powder.

Total proteins were solubilised by grinding the powder with extraction buffer (0.05 M sodium phosphate, pH 7.2; 0.01 M 2-mercaptoethanol; 1% SDS) in a hand-held homogeniser, at 5mg/ml. The suspension was heated at 95°C for 5 minutes, and centrifuged at 18 K for 20 minutes to remove insoluble material. The resulting clear supernatant contained about 1 mg/ml total protein. Electrophoresis of 25 μl on an SDS-PAGE gel (Laemmli, 1970) gave three major bands, including one at 21 kD, comprising approximately 30% of the total proteins. The 21 kD protein is presumed to be the polypeptide subunit of a major storage protein.

Characteristics of the Storage Polypeptide

The solubility characteristics of the 21 kD polypeptide was roughly defined by one or two quick experiments. Dialysis of the polypeptide solution against SDS-free extraction buffer rendered some polypeptides insoluble, as judged by their ability to pass through a 0.22 micron membrane, whereas the 21 kD polypeptide remained soluble. Only the 21 kD polypeptide was extracted from the acetone powder by water and dilute buffers, showing that this protein could be classed as an albumin.

Purification of the major polypeptide

The 21 kD polypeptide was purified by two rounds of gel filtration on a SUPEROSE-12 column of the PHARMACIA Fast Protein Liquid Chromatography system (FPLC), or by electroelution of bands after preparative electro- phoresis. (The words SUPEROSE and PHARMACIA are trade marks.) Concentrated protein extracts were made from 50 mg acetone powder per ml of extraction buffer, and 1-2 ml loaded onto 2 mm thick SDS-PAGE gels poured without a comb. After electrophoresis the gel was surface stained in aqueous

Coomassie Blue, and the major bands cut out with a scalpel. Gel slices were electroeluted into dialysis bags in electrophoresis running buffer at 15 V for 24 hours, and the dialysate dialysed further against 0.1% SDS. Samples could be concentrated by lyophilisation.

Example 2

Amino-acid Sequence Data from Protein

Protein samples (about 10 μg) were subjected to conventional N-terminal amino-acid sequencing. A 12 amino-acid sequence was obtained for the 21 kD protein, and this information was used to construct an oligonucleotide probe (Woods et al, 1982; Woods, 1984).

Example 3

Raising Antibodies to the 21 kD Polypeptide

Polyclonal antibodies were prepared using the methodology of Catty and Raykundalia (1988). The serum was aliquoted into 1 ml fractions and stored at -20°C.

Characterising Antibodies to the 21 kD Polypeptide

Serum was immediately characterised using the Ochterloney double-diffusion technique, whereby antigen and antibody are allowed to diffuse towards one another from wells cut in agarose in borate-saline buffer. Precipitin lines are formed where the two interact if the antibody 'recognises' the antigen. This test showed that antibodies to the 21 kD protein antigen had been formed.

The gamma-globulin fraction of the serum was partially purified by precipitation with 50% ammonium sulphate, solubilisation in phosphate-buffered saline (PBS) and chromatography on a DE 52 cellulose

ion-exchange column as described by Hill, 1984. Fractions containing gamma-globulin were monitored at 280 nm (OD_{280} of 1.4 is equivalent to 1 mg/ml gamma-globulin) and stored at -20°C.

The effective titre of the antibodies was measured using an enzyme-linked immunosorbant assay (ELISA). The wells of a polystyrene microtitre plate were coated with antigen (10-1000 ng) overnight at 4°C in carbonate coating buffer. Wells were washed in PBS-Tween and the test gamma globulin added at concentrations of 10, 1 and 0.1 μg/ml (approximately 1:100, 1:1000 and 1:10,000 dilutions). The diluent was PBS-Tween containing 2% polyvinyl pyrrolidone (PVP) and 0.2% BSA. Controls were preimmune serum from the same animal. Binding took place at 37°C for 3-4 hours. The wells were washed as above and secondary antibody (goat anti-rabbit IgG conjugated to alkaline phosphatase) added at a concentration of 1 μg/ml, using the same conditions as the primary antibody. The wells are again washed, and alkaline phosphatase substrate (p-nitrophenyl phosphate; 0.6 mg/ml in diethanol-amine buffer pH 9.8) added. The yellow colour, indicating a positive reaction, was allowed to develop for 30 minutes and the reaction stopped with 3M NaOH. The colour is quantified at 405 nm. More detail of this method is given in Hill, 1984. The method confirmed that the antibodies all had a high titre and could be used at 1 μg/ml concentration.

Example 4

Isolation of Total RNA from Immature Cocoa Beans

The starting material for RNA which should contain a high proportion of mRNA specific for the storage proteins was immature cocoa beans, at about 130 days after pollination. Previous work had suggested that synthesis of storage proteins was approaching its height by this date (Biehl et al, 1982). The beans are roughly corrugated and pale pinkish-purple at this age.

The initial requirement of the total RNA preparation from cocoa beans was that it should be free from contaminants, as judged by the UV spectrum, particularly in the far UV, where a deep trough at 230 nm (260 nm : 230 nm ratio is approximately 2.0) is highly diagnostic of clean RNA, and is intact, as judged by agarose gel electrophoresis of heat-denatured samples, which should show clear rRNA bands. A prerequisite for obtaining intact RNA is scrupulous cleanliness and rigorous precautions against RNases, which are ubiquitous and extremely stable enzymes. Glassware is customarily baked at high temperatures, and solutions and apparatus treated with the RNase inhibitor diethyl pyrocarbonate (DEPC, 0.1%) before autoclaving.

The most routine method for extraction of plant (and animal) RNA is extraction of the proteins with phenol/chloroform in the presence of SDS to disrupt protein-nucleic acid complexes, and inhibit the RNases which are abundant in plant material. Following phenol extraction the RNA is pelletted on a caesium chloride gradient before or after ethanol precipitation. This method produced more or less intact RNA, but it was heavily contaminated with dark brown pigment, probably oxidised polyphenols and tannins, which always co-purified with the RNA. High levels of polyphenols are a major problem in *Theobroma* tissues.

A method was therefore adopted which avoided the use of phenol, and instead used the method of Hall *et al.* (1978) which involves breaking the tissue in hot SDS-borate buffer, digesting the proteins with proteinase K, and specifically precipitating the RNA with LiCl. This method gave high yields of reasonably clean, intact RNA. Contaminants continued to be a problem and the method was modified by introducing repeated LiCl precipitation steps, the precipitate being dissolved in water and clarified by microcentrifugation after each step. This resulted in RNA preparations with ideal spectra, which performed well in subsequent functional tests such as *in vitro* translation.

Preparation of mRNA From Total RNA

The mRNA fraction was separated from total RNA by affinity chromatography on a small (1 ml) oligo-dT column, the mRNA binding to the column by its poly A tail. The RNA (1-2 mg) was denatured by heating at 65°C and applied to the column in a high salt buffer. Poly A+ was eluted with low salt buffer, and collected by ethanol precipitation. The method is essentially that of Aviv and Leder (1972), modified by Maniatis *et al* (1982). From 1 mg of total RNA, approximately 10-20 μg polyA+ RNA was obtained (1-2%).

In vitro Translation of mRNA

The ability of mRNA to support *in vitro* translation is a good indication of its cleanliness and intactness. Only mRNAs with an intact polyA tail (3' end) will be selected by the oligo-dT column, and only mRNAs which also have an intact 5' end (translational start) will translate efficiently. *In vitro* translation was carried out using RNA-depleted wheat-germ lysate (Amersham International), the *de novo* protein synthesis being monitored by the incorporation of [^{35} S]-methionine (Roberts and Paterson, 1973). Initially the rate of *de novo* synthesis was measured by the incorporation of [^{35} S]-methionine into TCA-precipitable material trapped on glass fibre filters (GFC, Whatman). The actual products of translation were investigated by running on SDS-PAGE, soaking the gel in fluor, drying the gel and autoradiography. The mRNA preparations translated efficiently and the products covered a wide range of molecular weights, showing that intact mRNAs for even the largest proteins had been obtained. None of the major translation products corresponded in size to the 21 kD polypeptide identified in mature beans, and it was apparent that considerable processing of the nascent polypeptide must occur to give the mature form.

Example 5

Identification of Precursor to the Mature Polypeptide by Immunoprecipitation

Because the 21 kD storage polypeptides was not apparent amongst the translation products of mRNA from developing cocoa beans, the technique of immunoprecipitation, with specific antibodies raised to the 21 kD polypeptide, was used to identify the precursors from the translation mixture. This was done for two reasons: first to confirm that the appropriate mRNA was present before cloning, and second to gain information on the expected size of the encoding gene.

Immunoprecipitation was by the method of Cuming *et al*, 1986. [^{35} S]-labelled *in vitro* translation products were dissociated in SDS, and allowed to bind with specific antibody in PBS plus 1% BSA. The antibody-antigen mixture was then mixed with protein A-SEPHAROSE and incubated on ice to allow the IgG to bind to protein A. The slurry was poured into a disposable 1 ml syringe, and unbound proteins removed by washing with PBS +1% NONIDET P-40. The bound antibody was eluted with 1M acetic acid and the proteins precipitated with TCA. The antibody-antigen complex was dissociated in SDS, and subject to SDS-PAGE and fluorography, which reveals which labelled antigens have bound to the specific antibodies.

The results showed that the anti-21 kD antibody precipitated a 23 kD precursor. The precursor size corresponded to a major band on the *in vitro* translation products.

Example 6

cDNA Synthesis From the mRNA Preparations

cDNA synthesis was carried out using a kit from Amersham International. The first strand of the cDNA is synthesised by the enzyme reverse transcriptase, using the four nucleotide bases found in DNA (dATP, dTTP, dGTP, dCTP) and an oligo-dT primer. The second strand synthesis was by the method of Gubler and Hoffman (1983), whereby the RNA strand is nicked in many positions by RNase H, and the remaining fragments used to prime the replacement synthesis of a new DNA strand directed by the enzyme *E. coli* DNA polymerase I. Any 3' overhanging ends of DNA are filled in using the enzyme T4 polymerase. The whole process was monitored by adding a small proportion of [^{32}P]-dCTP into the initial nucleotide mixture, and measuring the percentage incorporation of label into DNA. Assuming that cold nucleotides are incorporated at the same rate, and that the four bases are incorporated equally, an estimate of the synthesis of cDNA can be obtained. From 1 μg of mRNA approximately 140 ng of cDNA was synthesised. The products were analysed on an alkaline 1.4% agarose gel as described in the Amersham methods. Globin cDNA, synthesised as a control with the kit, was run on the same gel, which was dried down and autoradiographed. The cocoa cDNA had a range of molecular weights, with a substantial amount larger than the 600 bp of the globin cDNA.

Example 7

Cloning of cDNA into a Plasmid Vector by Homopolymer Tailing

The method of cloning cDNA into a plasmid vector was to 3' tail the cDNA with dC residues using the enzyme terminal transferase (Boehringer Corporation Ltd), and anneal into a *Pst*I-cut and 5' tailed plasmid (Maniatis *et al*, 1982 Eschenfeldt *et al*, 1987). The optimum length for the dC tail is 12-20 residues. The tailing reaction (conditions as described by the manufacturers) was tested

with a 1.5 kb blunt-ended restriction fragment, taking samples at intervals, and monitoring the incorporation of a small amount of [^{32}P]-dCTP. A sample of cDNA (70 ng) was then tailed using the predetermined conditions.

A dG-tailed plasmid vector (3'-oligo(dG)-tailed pUC9) was purchased from Pharmacia. 15 ng vector was annealed with 0.5 - 5 ng of cDNA at 58°C for 2 hours in annealing buffer: 5mM Tris-HCl pH 7.6; 1mM EDTA, 75 mM NaCl in a total volume of 50 μl. The annealed mixture was transformed into *E. coli* RRI (Bethesda Research Laboratories), transformants being selected on L-agar + 100 μg/ml ampicillin. Approximately 200 transformants per ng of cDNA were obtained. Transformants were stored by growing in 100 μl L-broth in the wells of microtitre plates, adding 100 μl 80% glycerol, and storing at -20°C.

Some of the dC tailed cDNA was size selected by electrophoresing on a 0.8% agarose gel, cutting slits in the gel at positions corresponding to 0.5, 1.0 and 1.5 kb, inserting DE81 paper and continuing electrophoresis until the cDNA had run onto the DE81 paper. The DNA was then eluted from the paper with high salt buffer, according to the method of Dretzen *et al* (1981).

Example 8

Construction of Oligonucleotide Probes for the 21 kD Gene

The N-terminus of the 21 kD polypeptide, as determined in Example 2 above, was

Ala-Asn-Ser-Pro-Leu-Asp-Thr-Asp-Gly-Asp-Glu.

From this the optimum region for synthesising a probe of 17 residues was as follows:

```
          Asp-Thr-Asp-Gly-Asp-Glu
5'   GAC ACC GAC GGC GAC GA   3'
       T   T   T   T   T
           A       A
           G       G
```

The 17-mer probe constructed is shown below the sequence: it is actually a mixture of 128 different 17-mers, one of which must be the actual coding sequence. Probe synthesis was carried out using an Applied Biosystems apparatus.

The 21 kD probe was purified by electrophoresis on a 20% acrylamide gel, the bands being detected by UV shadowing, and eluted by dialysing against water.

Example 9

Use of Oligonucleotides to Probe cDNA Library

The oligonucleotide probes were 5' end-labelled with gamma-[^{32}P] dATP and the enzyme polynucleotide kinase (Amersham International). The method was essentially that of Woods (1982, 1984), except that a smaller amount of isotope (15 μCi) was used to label about 40 ng probe, in 10 mM $MgCl_2$, 100 mM Tris-HCl, pH 7.6; 20 mM 2-mercaptoethanol.

The cDNA library was grown on GeneScreen (New England Nuclear) nylon membranes placed on the surface of L-agar + 100 μg/ml ampicillin plates. (The word GeneScreen is a trade mark.) Colonies were transferred from microtitre plates to the membranes using a 6 x 8 multi-pronged device, designed to fit into the wells of half the microtitre plate. Colonies were grown overnight at 37°C, lysed in sodium hydroxide and bound to membranes as described by Woods (1982, 1984). After drying the membranes were washed extensively in 3 x SSC/0.1% SDS at 65°C, and hybridised to the labelled probe, using a HYBAID apparatus from Hybaid Ltd, PO Box 82, Twickenham, Middlesex. (The word

HYBAID is a trade mark.) Conditions for hybridisation were as described by Mason & Williams (1985), a T_d being calculated for each oligonucleotide according to the formula:

T_d = 4°C per GC base pair + 2°C per AT base pair.
At mixed positions the lowest value is taken.

Hybridisation was carried out at T_d -5°C. Washing was in 6 x SSC, 0.1% SDS initially at room temperature in the HYBAID apparatus, then at the hybridisation temperature (T_d -5°C) for some hours, and finally at T_d for exactly 2 minutes. Membranes were autoradiographed onto FUJI X-ray film, with intensifying screens at - 70°C. (The word FUJI is a trade mark.) After 24 - 48 hours positive colonies stood out as intense spots against a low background.

Example 10

Analysis of Positive Clones for the 21 kD Polypeptide

Several positive clones were obtained with the 21 kD probe, and most of these contained an insert of 0.9 kb when digested with *PstI* (the original vector *PstI* site is re-created by the dG/dC tailing procedure). The inserts had the same restriction pattern, and are easily large enough to encode the 23 kD precursor, and it therefore seemed likely that they represented full-length clones. A map of the inset is shown in Figure 1.

The 0.9 kb *PstI* fragment was purified away from the vector by agarose gel electrophoresis onto DE81 paper (Dretzen *et al*, 1981), and about 500 ng was nick-translated using the Amersham nick-translation kit. The resulting probe was ~4 x 10^7 cpm and 10^6 cpm were used for the subsequent probing of the cDNA library, using the hybridisation method described by Wahl and Berger (1987). The conditions of 50% formamide and 42°C were used. Several more incomplete positive clones were obtained, which were useful in subsequent sequencing.

Example 11

Sequencing the Cloned Inserts

The sequencing strategy was to clone the inserts, and where appropriate subclones thereof, into the multiple cloning site of the plasmids pTZ18R/pTZ19R (Pharmacia). These plasmids are based on the better-known vectors pUC18/19 (Norrander *et al*, 1983), but contain a single-stranded origin of replication from the filamentous phage f1. When superinfected with phages in the same group, the plasmid is induced to undergo single-stranded replication, and the single-strands are packaged as phages extruded into the medium. DNA can be prepared from these 'phages' using established methods for M13 phages (Miller, 1987), and used for sequencing by the method of Sanger (1977) using the reverse sequencing primer. The superinfecting phage used is a derivative of M13 termed M13K07, which replicates poorly and so does not compete well with the plasmid, and contains a selectable kanamycin-resistance marker. Detailed methods for preparing single-strands from the pTZ plasmids and helper phages are supplied by Pharmacia. DNA sequence was compiled and analysed using the Staden package of programs (Staden, 1986), on a PRIME 9955 computer. (The word PRIME is a trade mark.)

Example 12

Features of the 21 kD cDNA, and Deduced Amino-acid Sequence of the 23 kD Precursor

The DNA sequence of the 21 kD cDNA, and the presumed amino-acid sequence of the encoded 23 kD precursor is shown in Figure 2. The cDNA is 917 bases, excluding the 3' poly A tail. The ATG start codon is at position 21, followed by an open reading frame of 221 codons, ending with a stop codon at position 684. This is followed by a 233-base untranslated region, which is relatively

AT-rich (60%) and has several stop codons in all three frames. There are two polyadenylation signals (AATAAA) at positions 753 and 887 (Proudfoot and Brownlee, 1976). At position 99 the sequence corresponding to the oligonucleotide probe is found, and at 167 the *Cla* site found experimentally.

The presumed 23 kD precursor polypeptide comprises 221 amino-acids and a molecular weight of 24003. The mature N-terminus is found at position 27, and the first 26 residues are highly hydrophobic, characteristic of a signal sequence recognised by the proteins responsible for translocating newly- synthesised proteins across membranes in the process of compartmentalisation (Kreil, 1981). The mature protein has 195 residues and a molecular weight of 21223, in good agreement with that deduced from polyacrylamide gels. The amino-acid composition of the mature protein is typical of a soluble protein with 24% charged residues and about 20% hydrophobic residues.

Homologies Between the 21 kD Protein and Other Known Proteins

Searching the protein identification resource (PIR) databank (National Biomedical Research Foundation, Washington DC) using the sequence matching program FASTP (Lipman and Pearson, 1985), showed a high degree of homology between the 21 kD protein and Kunitz-type protease and α-amylase inhibitors found in large amounts in the seeds of several species, particularly legumes and cereals. Examples, shown in Figure 3, include the barley α-amylase/subtilisin inhibitor, B-ASI (Svendsen *et al.* 1986), wheat α-amylase/ subtilisin inhibitor, W-ASI (Maeda, 1986), winged bean (*Pscophocarpus tetragonolobus*) chymotrypsin inhibitor, W-CI (Shibata *et al.* 1988), winged bean trypsin inhibitor, W-TI (Yamamoto *et al.* 1983), soybean trypsin inhibitor, S-TI (Koide and Ikenaka, 1973b), *Erythrina latissima* trypsin inhibitor, E-TI (Joubert *et al.* 1985).

All the Kunitz-type inhibitors are of a similar size and align along their entire length. Thus the 21 kD protein must belong to this general class.

Example 13

Expression of the 23 kD and 21 kD Polypeptides in E. coli

The DNA encoding the 23 kD and 21 kD polypeptides (ie. with and without the hydrophobic signal peptide) was subcloned into the *E. coli* expression vector, pJLA502 (Schauder et al, 1987) marketed by Medac GmbH, Postfach 303629, D-7000, Hamburg 36 (see Figure 4). The vector contains the strong lambda promoters, P_L and P_R, and the leader sequence and ribosome binding site of the very efficiently translated *E. coli* gene, *atp*E. It also contains a temperature-sensitive cI repressor, and so expression is repressed at 30°C and activated at 42°C. The vector has an *Nco*I site (containing an ATG codon: CCATGG) correctly placed with respect to the ribosome binding site, and foreign coding sequences must be spliced in at this point. The 23 kD coding sequence does not have an *Nco*I site at the initial ATG, so one was introduced by *in vitro* mutagenesis.

In vitro mutagenesis was carried out using a kit marketed by Amersham International, which used the method of Eckstein and co-workers (Taylor *et al*, 1985). After annealing the mutagenic primer to single-stranded DNA the second strand synthesis incorporates alpha-thio-dCTP in place of dCTP. After extension and ligation to form closed circles, the plasmid is digested with *Nci*I, an enzyme which cannot nick DNA containing thio-dC. Thus only the original strand is nicked, and subsequently digested with exonuclease III. The original strand is then resynthesised, primed by the remaining DNA fragments and complementing the mutated position in the original strand. Plasmids are then transformed into *E. coli* and checked by plasmid mini preparations.

An *Nco*I site was introduced into the 23 kD cDNA in plasmid pMS101 (in the vector pTZ19R, so that single-stranded DNA could readily be produced) using the mutagenic primer: 5' ACTTAACCATGGAGACC 3', to create the plasmid pMS106. The primer was chosen to avoid extensive hybridisation elsewhere in the plasmid.

The 23 kD coding region was cloned into the *E. coli* expression vector pJLA502 on an *Nco*I-*Eco*I fragment (pMS107). The coding region was then cloned back into pTZ19 on a *Xho*I (upstream of the *Nco*I) -*Eco*RI fragment. This creates a pTZ-23 kD plasmid (pMS108) which has eliminated the poly G/C region, likely to disrupt transcription between the T7 promoter in the vector and the coding region. *In vitro* transcription, using T7 RNA polymerase, produced abundant RNA which translated in a wheat germ system to give a 23 kD protein. This proves that a functional gene, capable of producing a protein of the anticipated size, is present on the plasmid.

The hydrophobic sequel sequence was deleted from plasmid pMS108 using a mutagenic primer designed to bind either side of the proposed deletion:

5' TGGAGACTGCCATGGCAAACTCTCCTGTG 3'

The resulting plasmid, pMS111, had retained an *Nco*I site at the ATG start, and the 21 kD coding region was subcloned into pJLA502 on an *Nco*I-*Bam*HI fragment (pMS113).

The two expression vectors were transformed into *E. coli* UT580. The transformed strains were grown in L-broth + ampicillin (100 μg/ml) at 30°C until log phase (OD_{610} = 0.5) and the temperature was then shifted to 42°C and samples taken at intervals. Samples were dissociated by boiling in SDS loading buffer, and run on SDS-PAGE gels. The proteins were electroblotted onto nitrocellulose membranes (Towbin et al, 1979) and Western blotting carried out using the anti-21 kD antibody prepared in Example 3 above (at 2 μg/ml) and as a secondary antibody, goat anti-rabbit -IgG conjugated to alkaline phosphatase (Scott *et al*, 1988).

For the vector pMS107 the antibody detected specific protein of molecular weight about 23 kD, but there were also smaller bands, including one at 21 kD suggesting that *E. coli* was partially cleaving the hydrophobic signal. The

largest amount of protein was seen after 18 hours, and was the equivalent of at least 1-2 mg/l. Controls containing only the vector gave no immuno-detectable proteins. For the vector pMS113 a similar result was obtained, except that only the 21 kD protein was seen: there was no evidence of higher expression in the absence of the signal sequence. However transforming the vectors into the protease-deficient strain CAG629 (Dr C.A. Gross) resulted in a much higher level of expression in both cases, in the order of 5-10 mg/l.

Example 14

Expression of the 21/23 kD Polypeptides in Yeast (Saccharomyces cerevisiae)

Two yeast expression vectors were used, both based on a yeast-*E. coli* shuttle vector containing yeast and *E. coli* origins of replication, and suitable selectable markers (ampicillin-resistance for *E. coli* and leucine auxotrophy for yeast). Both vectors contain the yeast pyruvate kinase (PK) promoter and leader sequence and have a *Hin*dIII cloning site downstream of the promoter. One vector, A, is designed for internal expression, and the other, B, for secreted expression, having a portion of the signal sequence of the yeast mating alpha-factor downstream of the promoter, with a *Hin*dIII site within it to create fusion proteins with incoming coding sequences. The vectors are illustrated in Figure 5.

To use the vectors effectively it is desirable to introduce the foreign coding region such that for vector A, the region from the *Hin*dIII cloning site to the ATG start is the same as the yeast PK gene, and for vector B, the remainder of the alpha-factor signal, including the lysine at the cleavage point. In practice this situation was achieved by synthesising two sets of *Hin*dIII - *Nco*I linkers to breach the gap between the *Hin*dIII cloning site in the vector and the *Nco*I at the ATG start of the coding sequence. For vector B, when the coding sequence is to be spliced to the yeast alpha-factor signal, the coding region of the 21 kD

polypeptide (ie. with the cocoa signal sequence removed) was used. The constructs are illustrated in Figure 6. For ease of construction of the yeast vectors, *Hin*dIII - *Nco*I linkers were first cloned into the appropriate pTZ plasmids, and *Hin*dIII - *Bam*III fragments containing linkers plus coding region cloned into the yeast vector.

The yeast expression plasmids were transferred into yeast spheroplasts using the method of Johnston (1988). The transformation host was the LEU^- strain AH22, and transformants were selected on leucine-minus minimal medium. LEU^+ transformants were streaked to single colonies, which were grown in 50 ml YEPD medium (Johnston, 1988) at 28°C for testing the extent and distribution of foreign protein. Cells were harvested from cultures in preweighed tubes in a bench-top centrifuge, and washed in 10 ml lysis buffer (200mM Tris, pH 8.1; 10% glycerol). The cell medium was reserved and concentrated 10-25 x in an AMICON mini concentrator. (The word AMICON is a trade mark.) The washed cells were weighed and resuspended in lysis buffer plus protease inhibitors (1mM phenyl methyl sulphonyl fluoride (PMSF); 1 μg/ml aprotinin; 0.5 μg/ml leupeptin) at a concentration of 1 g/ml. 1 volume acid-washed glass-beads was added and the cells broken by vortexing for 8 minutes in total, in 1 minute bursts, with 1 minute intervals on ice. After checking under the microscope for cell breakage, the mixture was centrifuged at 7000 rpm for 3 minutes to pellet the glass beads. The supernatant was removed to a pre-chilled centrifuge tube, and centrifuged for 1 hour at 20,000 rpm. (Small samples can be centrifuged in a microcentrifuge in the cold.) The supernatant constitutes the soluble fraction. The pellet was resuspended in 1 ml lysis buffer plus 10% SDS and 1% mercaptoethanol and heated at 90°C for 10 minutes. After centrifuging for 15 minutes in a microcentrifuge the supernatant constitutes the particulate fraction.

Samples of each fraction and the concentrated medium were examined by Western blotting. Plasmid pMS116, designed for internal expression, produced both 23 kD and 21 kD polypeptides in the soluble fraction of the cell lysate, and in the medium considerable amounts (2-5 mg/l) of the 21 kD polypeptide. Thus

the yeast is recognising the cocoa signal sequence and transporting the protein across the membrane, cleaving the signal during the process. The cleavage site appears to be correct, judging by the size of the final protein.

Plasmid pMS117, designed for secreted expression, gave a rather similar result with rather more 21 kD polypeptide in the medium. No evidence of the uncleaved polypeptide with the yeast alpha-factor signal still attached was found, either in the soluble or particulate fraction.

Example 15

Scale-up of Production of the 21 kD Protein in a 5 L Fermenter

To assess the productivity of the 21 kD protein from yeast AH22 containing the plasmid pMS117 under scale-up conditions the strain was grown in a 5L bioreactor manufactured by Life Technologies Inc. Like the small-scale growth experiments the medium used was YEPD, and the inoculum was 10 ml of a late log phase culture (OD_{600} 4.0). The aeration rate was 2L/min and the stirring speed 350 rpm, and to control the foaming caused by these aeration and stirring speeds 10 ml safflower oil was added. The cells were just entering log phase after 10 hours and by 15 hours the log phase was over with the disappearance of the glucose and accumulation of ethanol. However growth continued until the harvesting point at 60 hours, with the concomitant oxidation of the ethanol. The final biomass was 28 g/L wet weight, 7.3 g/L dry weight. Western blotting of the medium showed that 21 kD protein was exported to the medium slowly at first, but accumulated rapidly in late stationary phase rising to approximately 20-30 mg/L at the time of harvesting.

At the end of the experiment yeast cells were removed from the medium by cross-flow filtration through a 0.2 μm membrane, and the protein (or macromolecular) constituents in the medium were concentrated by cross-flow filtration through an ultra filtration membrane with a molecular weight cut-off

of 10 kD. The crossflow filtration apparatus was manufactured by Sartorius GmbH, Goettingen, Germany. The 21 kD protein can be further crudely purified by precipitation with 80% ammonium sulphate, followed by redissolving in water and dialysis.

Some enhancement of the yield was obtained by a batch feed process whereby the glucose levels were topped up to 2% from a concentrated solution as soon as the glucose levels had dropped below 0.1%. Four such additions were made at 16, 23, 34 and 37 hours, and growth continued until 58 hours. Improved yields of the 21 kD protein were obtained, up to 50 mg/L by the end of the experiment.

Example 16

Expression of the 23 kD/21 kD Protein in *Hansenula polymorpha*

The methylotrophic yeast *Hansenula polymorpha* offers a number of advantages over *Saccharamyces cerevisiae* as a host for the expression of heterologous proteins (EP-A-0173378 and Sudbery *et al*, 1988). The yeast will grow on methanol as sole carbon source, and under these conditions the enzyme methanol oxidase (MOX) can represent up to 40% of the total cell protein. Thus the MOX promoter is a very powerful one that can be used in a vector to drive the synthesis of heterologous proteins, and it is effective even as a single copy. This gives the potential to use stable integrated vectors. *Hansenula* can also grow on rich carbon sources such as glucose, in which case the MOX promoter is completely repressed. This means that cells containing the heterologous gene can be grown to a high density on glucose, and induced to produce the foreign protein by allowing the glucose to run out and adding methanol.

Constructs (pMY10 and pMY9) containing a 21 kD or 23 kD gene sandwiched between a MOX promoter and MOX terminator were made in the yeast episomal plasmid YEp13. Both contained a yeast secretion signal from

invertase spliced to the cocoa gene coding region, as illustrated in Figure 7. These constructs were transformed into *Hansenula* and both secreted the 21/23 kD protein into the medium under inducing conditions, although pMY10, containing the yeast signal but not the plant signal, was the most effective.

The *Hansenula* construct pMY10 has also been grown under scale-up conditions in a fermenter, and biomass yields of 45 g/L dry weight were obtained after induction with methanol. After induction the 21 kD protein was found in the medium in increasing amounts up to 50 mg/L.

E. coli Strains

RR1 $F^-v_B^-M_B$ *ara*-14 *pro*A2 *leu*B6 *lac*Y1 *gal*K2 *vps*L20 (str^r) *xyl*-5 *mtl*-1 *sup*E44 $^-$

CAG629 lac_{am} tvp_{am} pho_{am} *htp*R_{am} *mal* *rps*L *lon* *sup*C_{ts}

UT580 (*lac-pro*) *sup*E *thi* *hsd*D5 / F'*tra* D36 *pro*A^+B^+ *lac*I^q *lac*Z M15

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CLAIMS

1. A 23kD protein of *Th. cacao*, or a fragment thereof.

2. A 21kD protein of *Th. cacao*, or a fragment thereof.

3. A protein as claimed in claim 1 or 2 having at least part of the sequence shown in Figure 2.

4. A fragment as claimed in claim 1, 2, or 3 which comprises at least four amino acids.

5. A protein or fragment as claimed in any one of claims 1 to 4 which is recombinant.

6. Recombinant or isolated nucleic acid coding for a protein or fragment as claimed in any one of claims 1 to 5.

7. Nucleic acid as claimed in claim 6 which is DNA.

8. Nucleic acid as claimed in claim 7 having at least part of the sequence shown in Figure 2.

9. Nucleic acid as claimed in claim 6, 7 or 8, which is in the form of a vector.

10. Nucleic acid as claimed in claim 9, wherein the vector is an expression vector and the protein- or fragment-coding sequence is operably linked to a promoter.

11. Nucleic acid as claimed in claim 10, wherein the expression vector is a yeast expression vector and the promoter is a yeast pyruvate kinase (PK) promoter.

12. Nucleic acid as claimed in claim 10, wherein the expression vector is a bacterial expression vector and the promoter is a strong lambda promoter.

13. Nucleic acid as claimed in claim 10, 11 or 12, comprising a signal sequence.

14. A host cell comprising nucleic acid as claimed in any one of claims 9 to 13.

15. A host cell as claimed in claim 14 which is *Saccharomyces cerevisiae*.

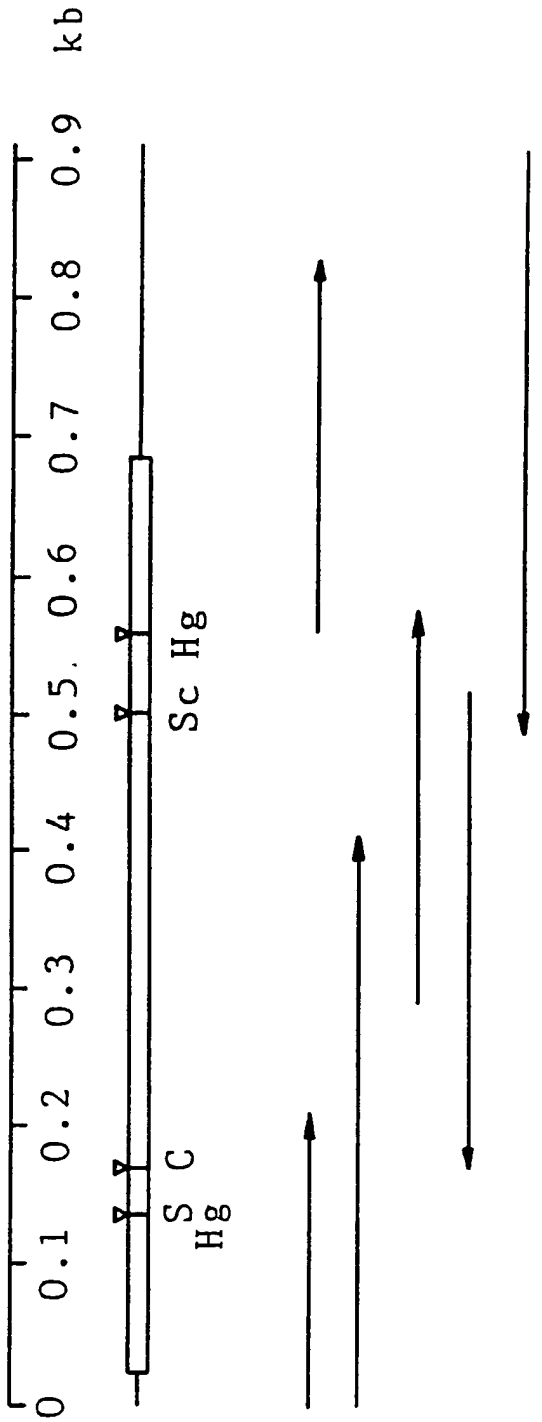
16. A host cell as claimed in claim 14 which is *E. coli*.

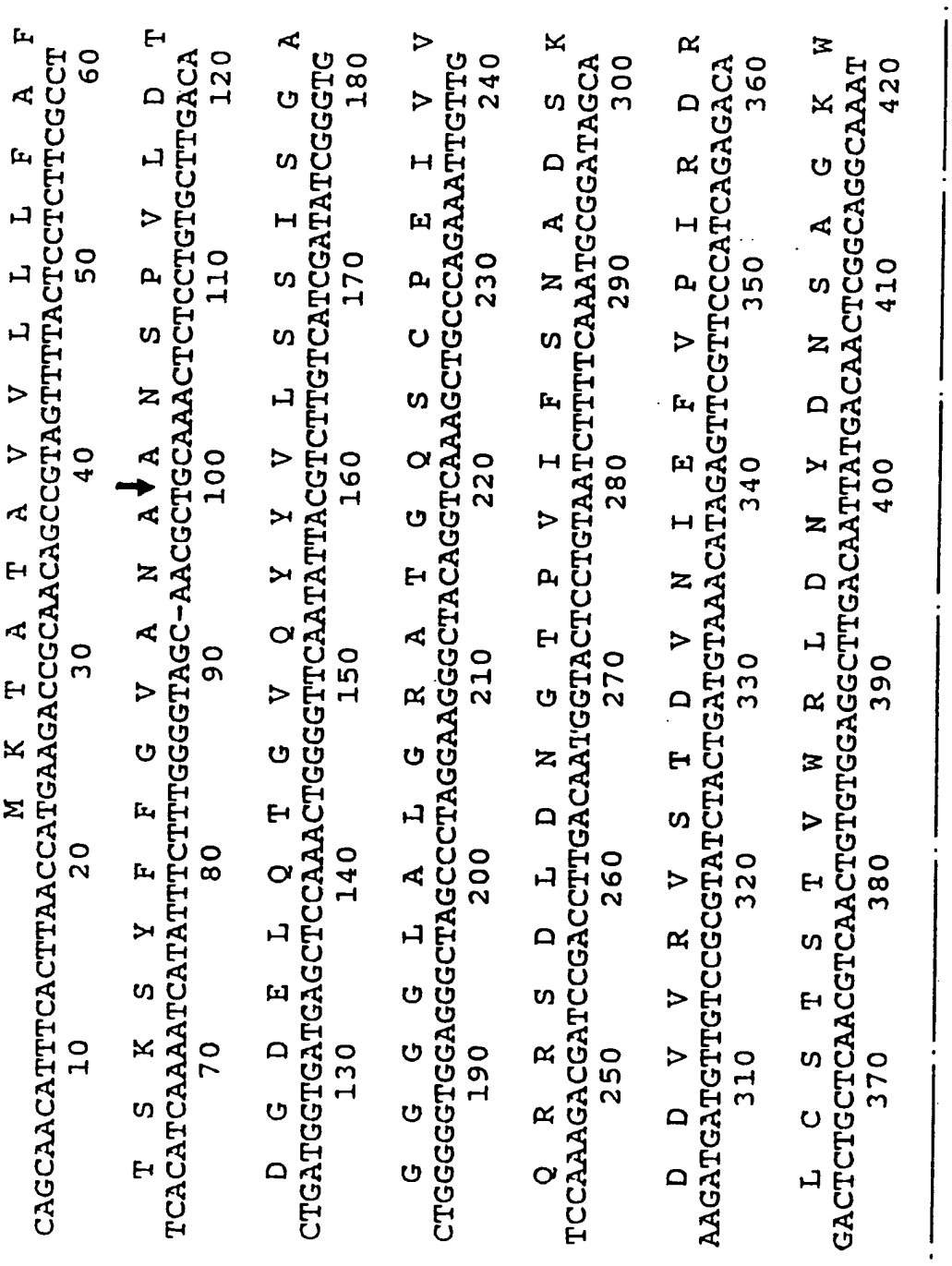
17. A process for the preparation of a protein or fragment as claimed in any one of claims 1 to 4, the process comprising coupling successive amino acids by peptide bond formation.

18. A process for the preparation of a protein or fragment as claimed in any one of claims 1 to 4, the process comprising culturing a host cell as claimed in claim 14, 15 or 16.

19. A process for the preparation of nucleic acid as claimed in any one of claims 6 to 13, the process comprising coupling together successive nucleotides and/or ligating oligo- or poly-nucleotides.

FIG. 1

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CAGCAACATTTCACTTAACCATGAAGACCGCAACAGCCGTAGTTTTACTCCTCTTCGCCT
10 20 30 40 50 60
T S K S Y F F G V A N A A N S P V L D T
TCACATCAAAATCATATTTCTTTGGGGTAGC-AACGCTGCAAACTCTCCTGTGCTTGACA
70 80 90 100 110 120
D G D E L Q T G V Q Y Y V L S S I S G A
CTGATGGTGATGAGCTCCAAACTGGGGTTCAATATTACGTCTTGTCATCGATATCGGGTG
130 140 150 160 170 180
G G G G L A L L G R A T G Q S C P E I V V
CTGGGGGTGGAGGGCTAGCCCTAGGAAGGGCTACAGGTCAAGCTGCCCAGAAATTGTTG
190 200 210 220 230 240
Q R R S D L L D N G T P V I F S N A D S K
TCCAAAGACGATCCGACCTTGACAATGGTACTCCTGTAATCTTTTCAAATGCGGATAGCA
250 260 270 280 290 300
D D V V R V S T D V N I E F V P I R D R
AAGATGATGTTGTCCGCGTATCTACTGATGTAAACATAGAGTTCGTTCCCATCAGACA
310 320 330 340 350 360
L C S T S T V W R L D N Y D N S A G K W
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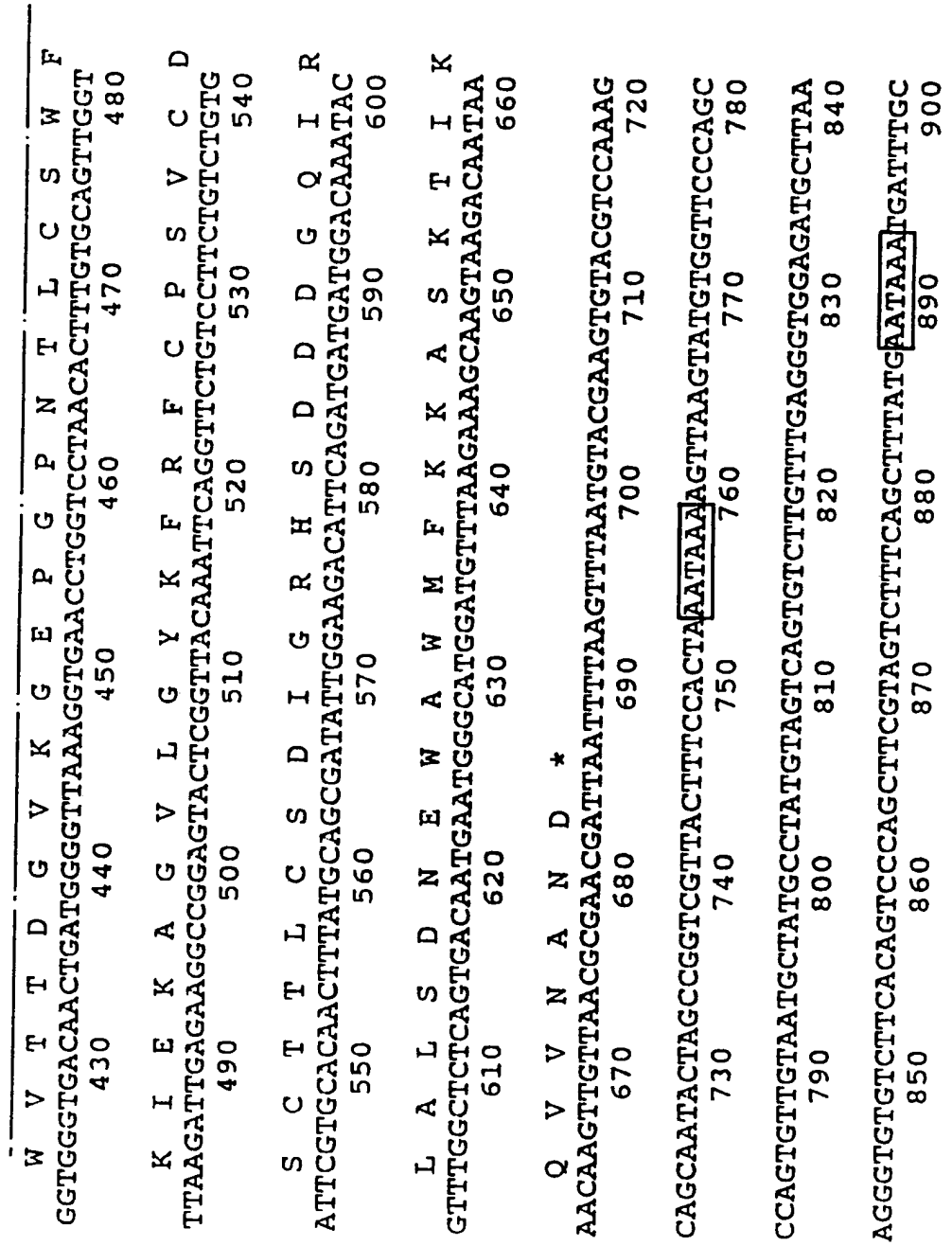
370 380 390 400 410 420

FIG. 2A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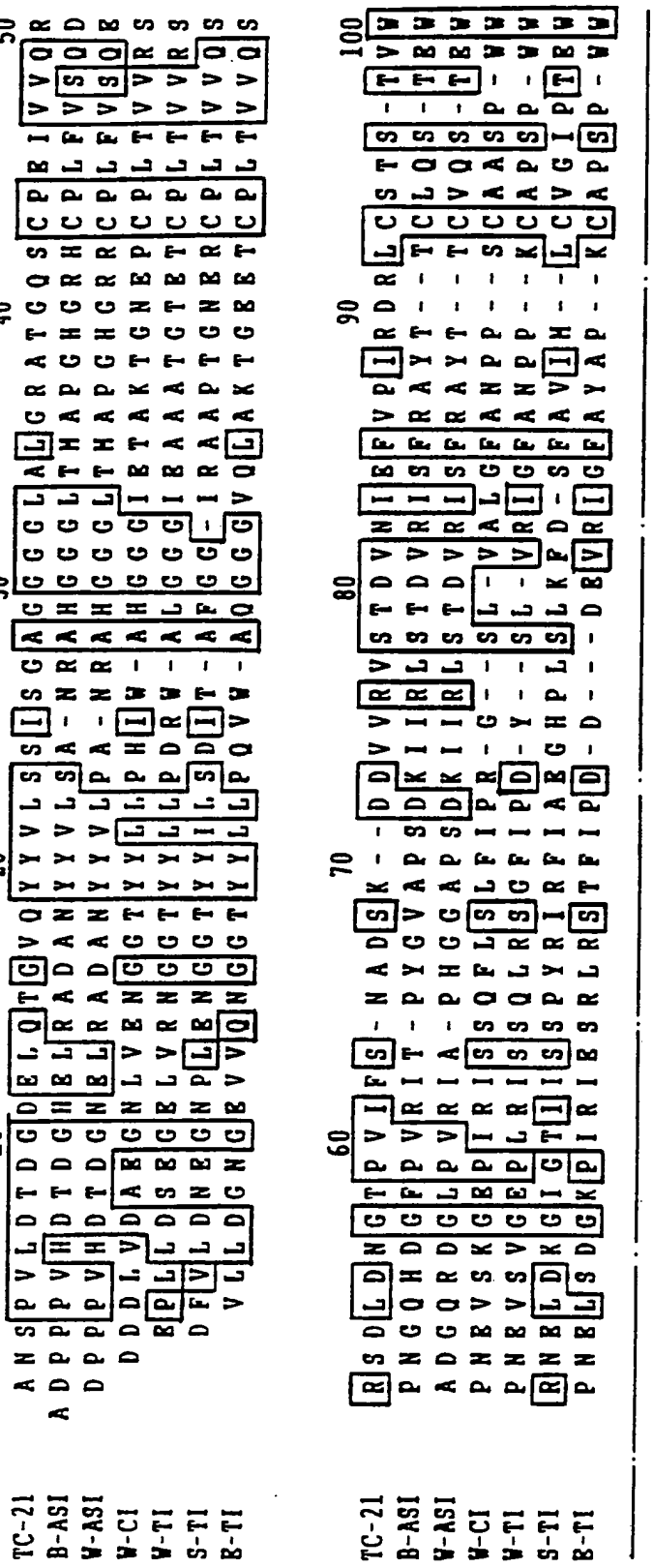
430 440 450 460 470 480
K I E K A G V L G Y K F R F C P S V C D
TTAAGATTGAGAAGGCCGGAGTACTCGGTTACAAATTCAGGTTCTGTCCTTCTGTCTGTG
490 500 510 520 530 540
S C T T L C S D I G R H S D D D G Q I R
ATTCGTGCACAACTTTATGCAGCGATATTGGAAGACATTCAGATGATGGACAAATAC
550 560 570 580 590 600
L A L S D N E W A W M F K K A S K T I K
GTTTGGCTCTCAGTGACAATGAATGGGCATGGATGTTTAAGAAAGCAAGTAAGACAATAA
610 620 630 640 650 660
Q V V N A N D *
AACAAGTTGTTAACGCGAACGATTAATTTTAAGTTTAATGTACGAAGTGTACGTCCAAAG
670 680 690 700 710 720
CAGCAATACTAGCCGGTCGTTACTTTCCACTAATAAAAGTTAAGTATGTGGTTCCCAGC
730 740 750 760 770 780
CCAGTGTTGTAATGCTATGCCTATGTAGTCAGTGTCTTGTTTGAGGGTGGAGATGCTTAA
790 800 810 820 830 840
AGGGTGTGTCTTCACAGTCCCAGCTTCGTAGTCTTTCAGCTTTATGAATAAATGATTTGC
850 860 870 880 890 900
CTCTTGCCTCTTTTATT
910

FIG. 2B

FIG. 3A



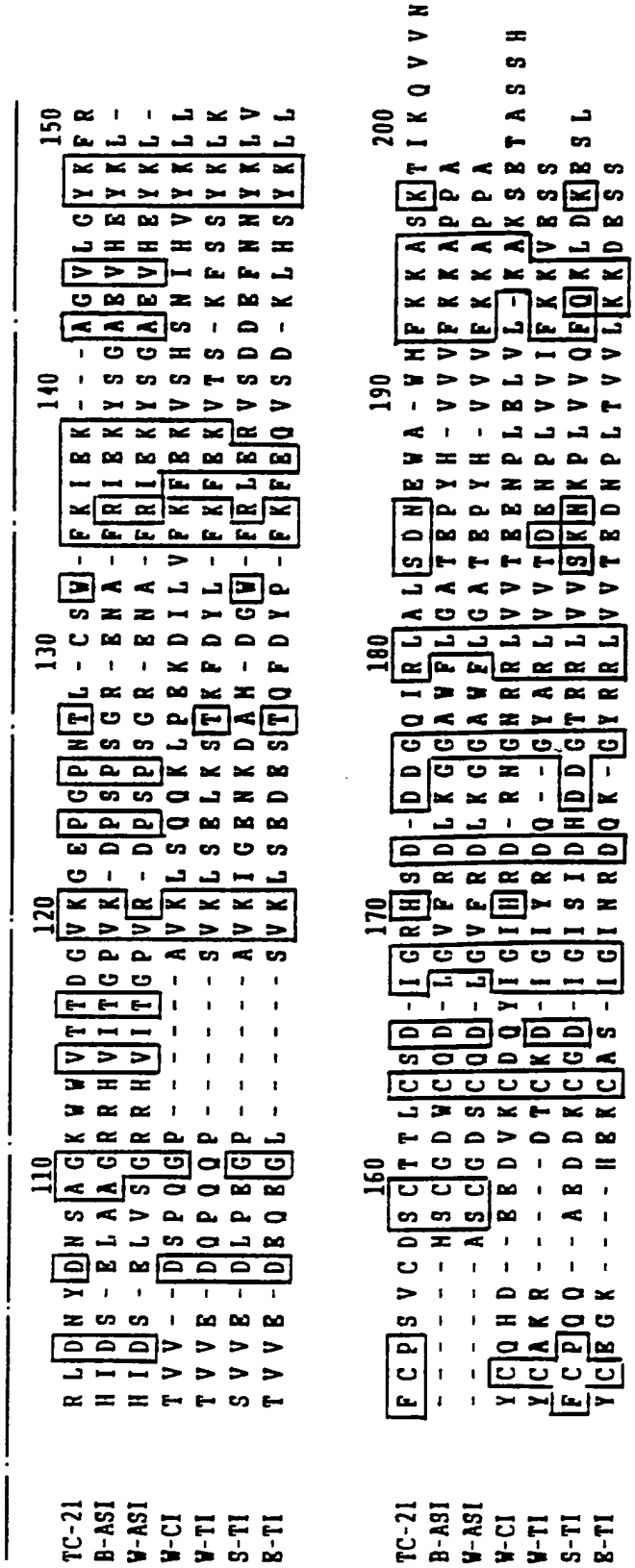


FIG. 3B

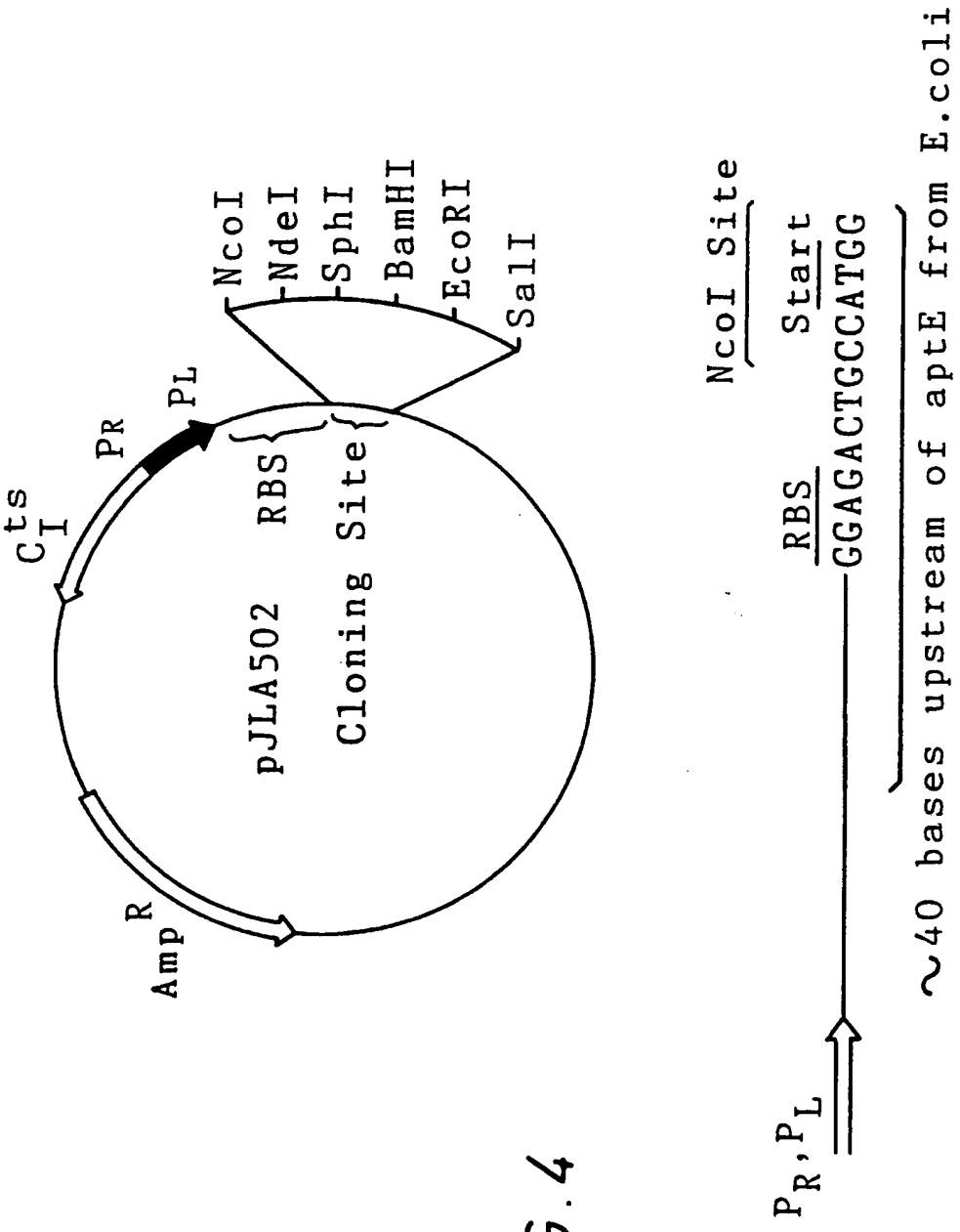

NcoI
NdeI
SphI
BamHI
EcoRI
SalI
PL
PR
cts I
RBS
Cloning Site
pJLA502
R
Amp
NcoI Site
Start
RBS
GGAGACTGCCATGG
~40 bases upstream of aptE from E.coli
PR, PL

FIG. 4

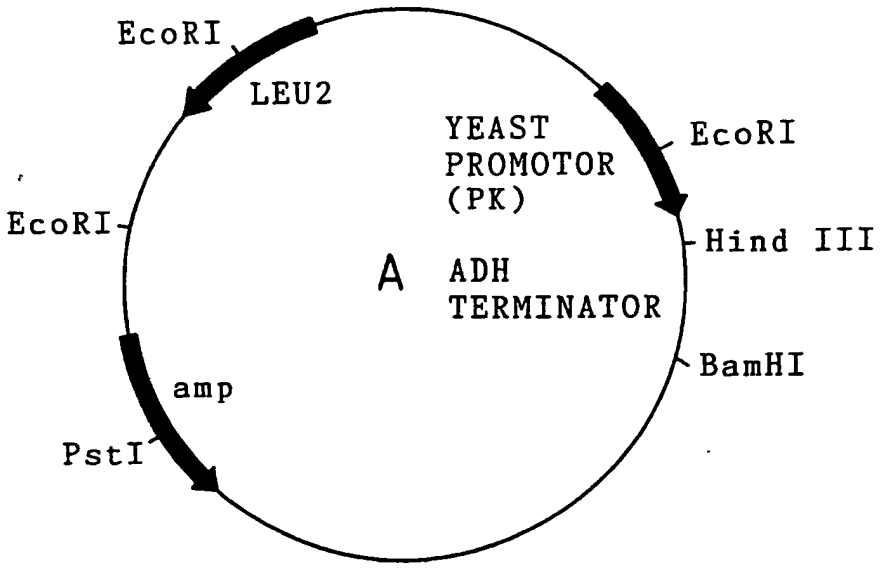

EcoRI
LEU2
YEAST
PROMOTOR
(PK)
EcoRI
EcoRI
Hind III
A
ADH
TERMINATOR
BamHI
amp
PstI

FIG. 5

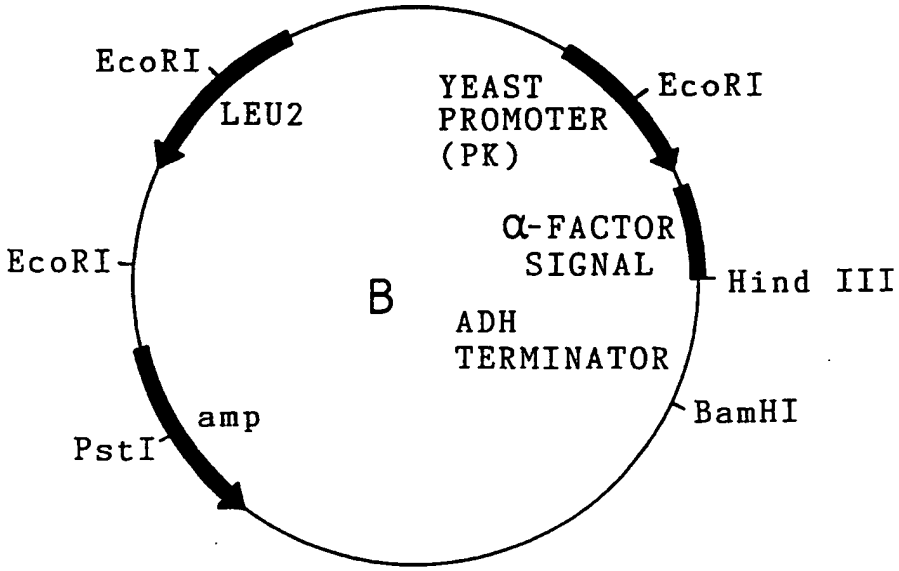

EcoRI
LEU2
YEAST
PROMOTER
(PK)
EcoRI
α-FACTOR
SIGNAL
EcoRI
B
Hind III
ADH
TERMINATOR
BamHI
amp
PstI

FIG. 6A

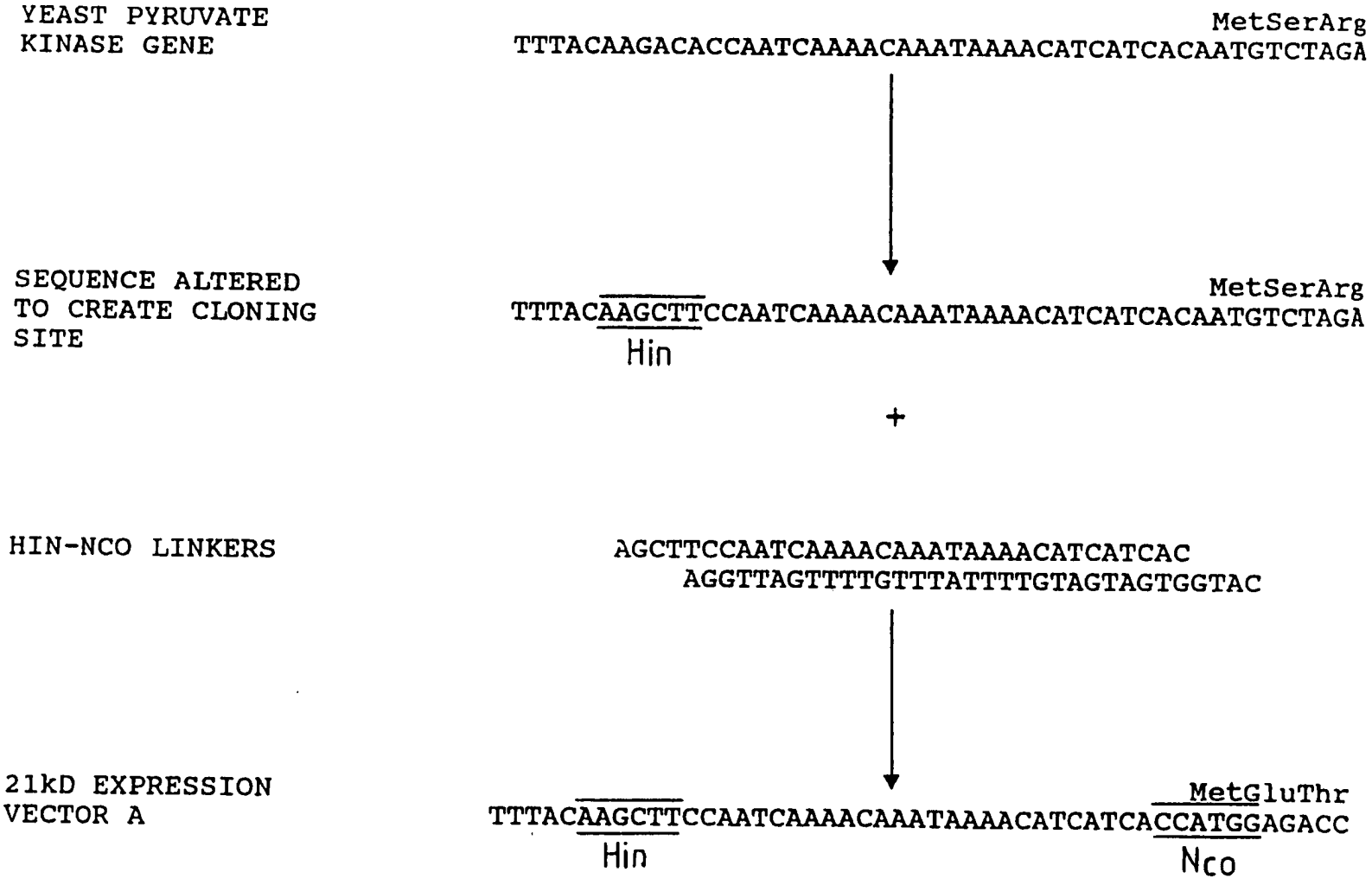
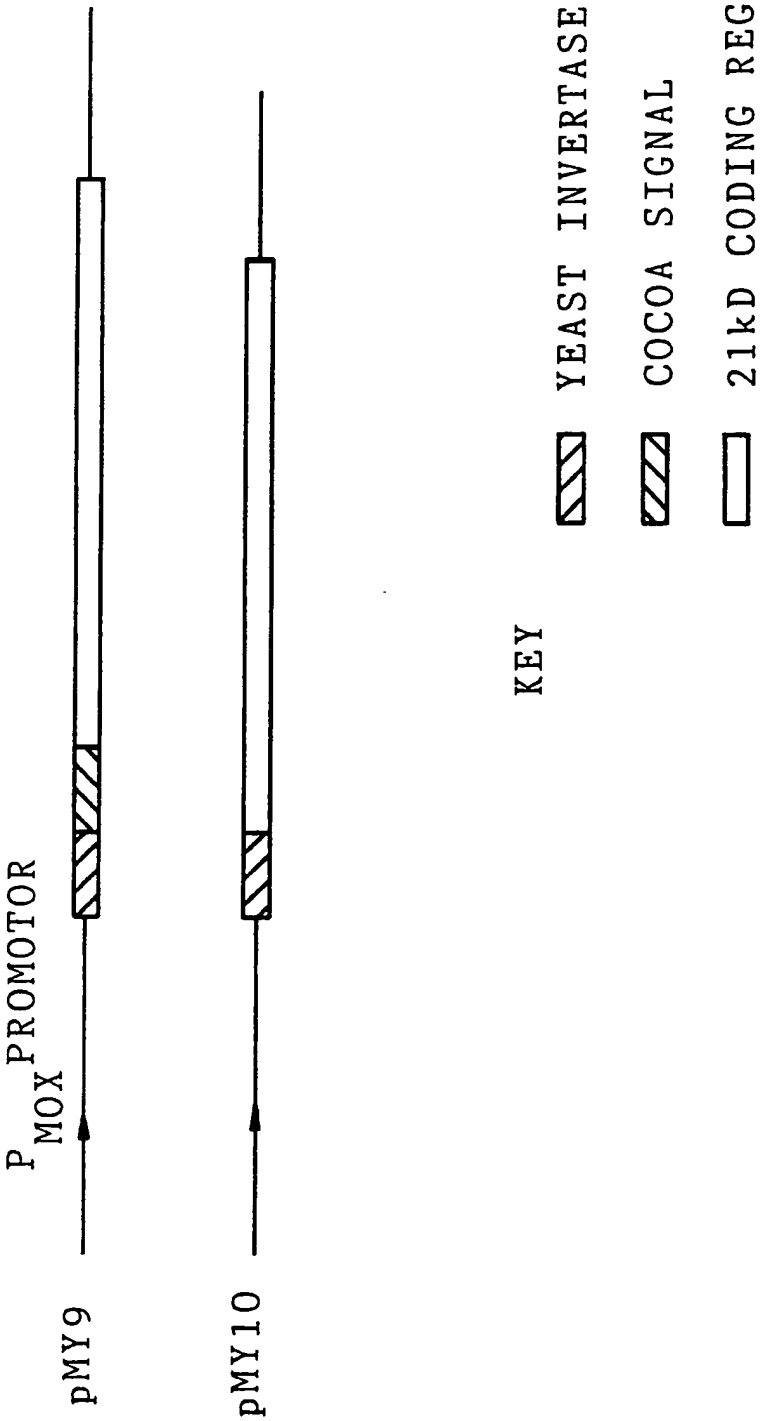

YEAST PYRUVATE KINASE GENE
MetSerArg
TTTACAAGACACCAATCAAAACAAATAAAACATCATCACAATGTCTAGA
SEQUENCE ALTERED TO CREATE CLONING SITE
MetSerArg
TTTACAAGCTTCCAATCAAAACAAATAAAACATCATCACAATGTCTAGA
Hin
+
HIN-NCO LINKERS
AGCTTCCAATCAAAACAAATAAAACATCATCAC
AGGTTAGTTTTGTTTATTTTGTAGTAGTGGTAC
21kD EXPRESSION VECTOR A
MetGluThr
TTTACAAGCTTCCAATCAAAACAAATAAAACATCATCACCATGGAGACC
Hin
Nco

FIG. 6B

	1 231
YEAST ALPHA-FACTOR SIGNAL SEQUENCE	Met----GluGlyValSerLeuAspLysArgGlu ATG----GAAGGGGTAAGCTTGGATAAAAGAGAG Hin
HIN-NCO LINKERS	AGCTTGGATAAAAGAGC ACCTATTTTCTCGGTAC
IN-PHASE FUSION OF 21KD CODING REGION	Met---GluGlyValSerLeuAspLysArgAlaMetAlaAsn ATG---GAAGGGGTAAGCTTGGATAAAAGAGCCATGGCAAAC Hin Nco

FIG. 7


KEY
YEAST INVERTASE SIGNAL
COCOA SIGNAL
21kD CODING REGION
P MOX PROMOTOR
pMY9
pMY10

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 91/00913

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/29 ; C07K13/00 ; C12N1/19 ; C12N1/21

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	J. SCI. FOOD AGRIC. vol. 33, 1982, pages 1291 - 1304; BIEHL, B., ET. AL.: 'Vacuolar storage proteins of cocoa seeds and their degradation during germination and fermentation ' see the abstract	1-5
X	J. FOOD SCIENCCE vol. 50, 1985, pages 946 - 950; FRITZ, P. J. ,ET. AL.: 'Cocoa seeds : changes in protein and polysomal RNA during development ' see the whole document	1-5
Y		6-19

-/--

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
27 SEPTEMBER 1991	29. 10. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MADDOX A.D.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED T BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	ABSTR. PAP. AM. CHEM. SOC. vol. 188, 1984, BIOL 148 WILSON, M. R., ET. AL.: 'Cocoa Theobroma cacao seed complementary DNA library ' see the abstract 148	6-19
X	PLANT MOL BIOL vol. 12, 1989, pages 673 - 682; LEAH R.,ET.AL.: 'The bifunctional alpha-amylase/ subtilisin inhibitor of barley: nucleotide sequence and patterns of seed-specific expression ' see the whole document	3-1013, 14,16,18
X	EP,A,297 834 (ABI BIOTECHNOLOGY) January 4, 1989 see page 4 - page 6; claims 10-13; figure 1	3-10, 13-15,18
P,X	PLANTA vol. 183, no. 4, 1991, pages 528 - 535; SPENCER M. E., ET. AL.: 'Cloning and sequencing of the cDNA encoding the major albumin of Theobroma cacao ' see the whole document	1-10,13, 14,16,19
A	CAFE CACAO THE vol. 34, no. 1, January 1990, pages 23 - 26; PETTIPHER G. L.: 'The extraction and partial purification of cocoa storage proteins ' see the whole document	1-5

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

GB 9100913
SA 48331

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
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27/09/91

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-297834	04-01-89	JP-A- 1157385	20-06-89
		US-A- 4910297	20-03-90